

DEVELOPMENT OF NUCELLAR PLANTS FROM UNPOLLINATED AND UNFERTILISED OVULES OF THE WASHINGTON NAVEL ORANGE *IN VITRO**

James Button and Chris H. Bornman

(Department of Botany, University of Natal, Pietermaritzburg)

SHORT COMMUNICATION

ABSTRACT

Previously, pollination and fertilisation have been regarded as essential prerequisites for the initiation of nucellar embryoids in *Citrus*. However, a recent attempt at inducing embryoids and the subsequent differentiation of entire plants from nucellar isolates of ovules of the Washington navel orange has been successfully carried out for the first time.

The induction of pseudobulbils and the subsequent differentiation of embryoids was most effective on a basal nutrient medium supplemented with 40 mg/l adenine and 400 mg/l malt extract. Then, entire plantlets were differentiated from these embryoids when transferred to a basal medium containing 1 mg/l gibberellic acid.

UITTREKSEL

ONTWIKKELING, *IN VITRO*, VAN NUSELLUS PLANTE UIT ONBESTUIFDE EN ONBEVRUGTE SAADKNOPPE VAN DIE WASHINGTON NAVELLEMOEN. Tot op hede is bestuiwing en bevrugting as noodsaaklike voorvereistes beskou vir die daarstel van nusellêre embrioeë in *Citrus*. Die eerste suksesvolle poging om embrioeë te induseer, gevolg deur differensiasie van volledig ontwikkelde plante uit die nusellêre isolate van die saadknoppe van die Washington nawellemoen, word beskryf. Die induksie, eerstens, van sogenaamde skynbolle en die differensiasie van die embrioeë is verkry op 'n basiese voedingsmedium met toevoeging van 40 mg/l adenien en 400 mg/l moustekstrak. Heel plantjies is daaropvolgens uit dié embrioeë gedifferensieër deur laasgenoemde oor te plant op 'n basiese medium wat 1 mg/l gibberelliensuur bevat het.

INTRODUCTION

In addition to meristem culture, the culture of haploid plants from anthers (Guha and Maheshwari, 1967), and cell hybridisation by protoplasmic fusion (Nickell and Torrey, 1969), the establishment of clonal plants of nucellar origin holds great potential significance for crop improvement. *Citrus* nucellar-lines are virus-free, more vigorous, usually higher-producing, and are longer-lived than the parent-line trees although their genetic constitutions, except possibly for occasional somatic mutations, are identical.

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Normally, the Washington navel orange is completely seedless. This results from degeneration of (a) the microsporogenous tissue before the first meiotic divisions occur (Webber, 1894, 1930; Osawa, 1912), with the result that no pollen whatsoever is formed, and (b) the megasporocyte or the megagametophyte (Osawa, 1912). Occasional seeds, 10 in over 25,000 fruits (Shamel, 1918), may be formed as a result of cross-pollination but this rarely occurs as *Citrus* pollen is strictly entomophilous.

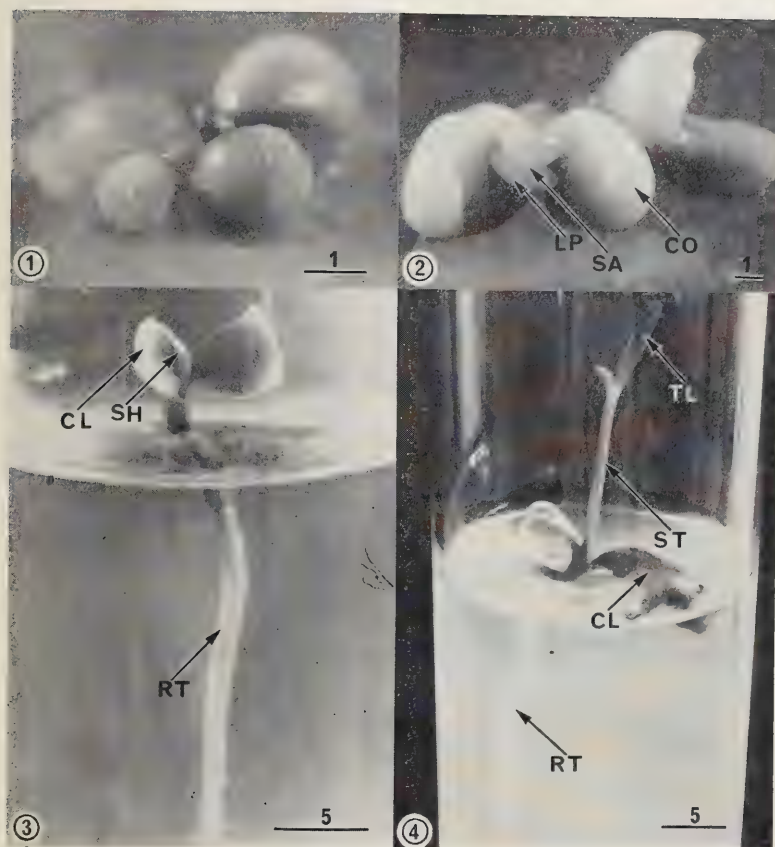
Frost and Soost (1968) concluded that pollination and fertilisation are usually, but perhaps not invariably necessary for the initiation of nucella embryos, *in vivo*. Pollination and fertilisation appear to provide an essential stimulus to the nucellus via the zygotic embryo, inducing it to produce adventive embryos. Once this triggering stimulus has been received by the nucellus it appears that the subsequent abortion of the zygotic embryo does not affect further development of the nucellar embryos.

Numerous attempts have been made to culture, and induce differentiation of ovules, zygotic embryos, and nucellar embryos of *Citrus in vitro* (Stevenson, 1956; Ohta and Furusato, 1957; Rangaswamy, 1958a, 1958b, 1959, 1961; Rangan, Murashige and Bitters, 1968, 1969). Sabharwal (1962) was unable to induce development of nucellar embryos *in vitro* unless these had been initiated before excision of the nucelli. The inference here of the necessity for a prior stimulus, was supported more recently by Rangaswamy (1970) who is of the opinion that pollination and fertilisation are essential prerequisites for the initiation and development of nucellar embryos *in vitro*. Rangan *et al.* (1968, 1969) are the only workers who have succeeded in triggering the initiation of adventive embryos in the nucellus of some monoembryonic *Citrus* varieties. They used excised nucellar isolates from developing seeds which had been fertilised with compatible pollen of *Poncirus trifoliata*.

As far as could be ascertained, nucellar embryoids have never been successfully initiated and differentiated from unfertilised, unpollinated ovules where no adventive embryos had been stimulated. Considering the advantages of producing nucellar plants from unpollinated, unfertilised ovules, particularly of monoembryonic cultivars, a series of experiments were undertaken in an attempt to initiate such plants by the careful control and manipulation of basal nutrient media, hormones, and other plant growth regulating substances.

MATERIALS AND METHOD

Fruits were picked from a Washington navel orange tree at the Ukulinga Research Station, Pietermaritzburg, about eight weeks after anthesis. Careful microscopic examination indicated that the ovules showed no signs of either zygotic or adventive embryo development. Nucellar isolates, ca. 0.1 mm³,



FIGS. 1—4.

Stages in development of plantlet from nucellus of unpollinated ovule of Washington navel orange. Fig. 1. Pseudobulbils, 30 days after transfer of nucellar isolates to culture medium containing 40 mg/l adenine and 400 mg/l malt extract. Fig. 2. Differentiated embryooids at 60 days. Fig. 3. Germinating embryooid, 10 days, after transfer to culture medium containing 1 mg/l gibberellic acid. Fig. 4. Entire plant, 28 days after transfer. CO, cotyledon; CL, cotyledonary leaf; LP, leaf primordium; RT, root; SA, shoot apex; SH, shoot; ST, stem; and TL, true leaf. Scale lines in mm.

were excised aseptically with the aid of a dissecting microscope and placed on a basal nutrient medium (BM) composed of the inorganic salts of White (Rangaswamy, 1961) or Murashige and Skoog (1962). The following organic substances were added (in mg/l):

glycine, 5.0; thiamine hydrochloride, 0.3;
pyridoxin hydrochloride, 0.05; niacin, 1.0;
calcium pantothenate, 0.03; myo-inositol, 100;
sucrose, 4.0×10^4 ; and agar, 10^4

The pH of the complete medium was adjusted to 5.6 before autoclaving for 15 minutes at 1.05 kg/cm^2 .

Plant growth substances. From extensive work in progress in our tissue culture laboratory on the monoembryonic varieties—Ellendale mandarin and Shaddock, various types, concentrations, combinations, and ratios of auxins, cytokinins and gibberellins were used, and it became clear that these plant hormones, *per se*, do not stimulate the nucellus into forming embryoids. Other organic additives used in these experiments included yeast extract, coconut milk, malt extract, ascorbic acid, casein hydrolysate, adenine and adenine sulphate.

The only additives which showed promise were coconut milk, adenine, adenine sulphate, ascorbic acid and malt extract and it was therefore decided to test their efficacy, alone and in combination, in inducing embryoids in nucellar isolates of unfertilised and unpollinated Washington navel ovules at the following levels:

coconut milk (CM), 15% v/v; adenine (AD), 40 mg/l;
adenine sulphate (AS) 40 mg/l; ascorbic acid (AA), 40 mg/l;
and malt extract (ME), 400 mg/l.

Culture conditions. The nucellar isolates were cultured in test tubes fitted with Cap-O-Test caps lined with non-absorbent cotton wool and held at $24 \pm 2^\circ\text{C}$ under a photoperiod of 16 hours. Light was supplied from Gro-Lux fluorescent tubes at an intensity of 10^3 lux .

RESULTS AND DISCUSSION

Initiation and development of nucellar embryoids. Rangan *et al.* (1968, 1969), working with fertilised ovules, reported a response in nucellar embryoid initiation from a medium which included either the auxin, naphthaleneacetic acid, in combination with orange juice and adenine sulphate, or malt extract only.

Embryoids have been induced of course, in free-cell cultures as well as from tissue explants taken from many plant organs but the necessity and role of substances such as casein hydrolysate, auxins, and coconut milk in the induction and differentiation of embryoids is still not clear (Konar and Nataraja, 1965a, b; Johri and Sehgal, 1966; Sussex and Frei, 1968).

The first signs of growth in our nucellar isolates were the appearance of *pseudobulbils* (Fig. 1), a term used by Rangaswamy (1958a). These were first observed about 4 weeks after transplanting, being preceded by very little callus formation. Pseudobulbils gradually developed into embryoids (Fig. 2). Although

there is inconsistency in the literature, an 'embryoid' is generally accepted as being an organ similar in structure to an embryo but derived asexually. No growth occurred on BM alone, and occurred to a limited extent only on BM + AA. However, when on BM + AD or BM + AS or BM + ME, growth was rapid with numerous embryoids developing from the pseudobulbils. Basal medium + CM was not as effective, but when used in combination with AD, AS and/or ME, it did increase the incidence of pseudobulbil and embryoid formation. Basal medium + AD + ME yielded the best results by not only initiating the largest number of pseudobulbils but also by producing most embryoids per nucellar isolate. Further differentiation of the embryoid masses into discrete organs at this stage—about 10 weeks—appeared impossible on any of the above media.

Differentiation of embryoids and development of plantlets. We did not wish to assume that because the plant hormones auxin, cytokinin and gibberellin had no apparent effect on embryoid initiation in the nucellar isolates, they would be equally ineffective in inducing the differentiation of complete organs, despite the fact that Steward (1969) had shown that auxin prevents differentiation of entire plants from embryoids in some free-cell cultures.

Consequently, the embryoid masses were subdivided and, as far as was possible, only single embryoids were transferred to the following media:

BM + 0.1 mg/l indoleacetic acid (IAA);

BM + 0.1 mg/l kinetin (CK); and BM + 1.0 mg/l
gibberellic acid (GA_3)

Gibberellic acid was included since in previous experiments we had found it to promote the formation of roots. This was somewhat unexpected since GA_3 is not generally associated with root initiation. Rangaswamy (1961) did, however, find a similar response in his nucellar cultures. Within one week of transplanting the embryoids more than 60 per cent on BM + GA_3 had developed roots (Fig. 3). No roots were formed in the other treatments until about three weeks after transplanting, and then only in a limited number.

The emergence of the first true leaves occurred about three weeks after transplanting (two weeks after the radicle appeared), but only in plantlets on BM + GA_3 (Fig. 4). At the time of writing (four weeks after transplanting) BM + GA_3 is still the only medium on which entire plantlets have been formed. It is possible, however, that entire plantlets will be formed on BM + IAA and BM + CK. But it is doubtful whether any will be formed on BM alone as further slow generation of embryoids is occurring.

Isolated, single embryoids differentiate more readily than aggregates following transplantation.

CONCLUSION

What appears to be the first successful attempt at producing nucellar citrus plants from unpollinated and unfertilised ovules, has been achieved in our laboratory. An important practical application of this technique is that it can be used to import seedless citrus varieties without the danger of introducing new viral diseases, thus obviating the necessity of subjecting vegetative material to long periods of quarantine. In addition, it can be used to rapidly propagate a large number of clonal nucellar trees from say a single fruit of a seedless *Citrus* variety since numerous embryoids and plantlets can be differentiated and grown from each original nucellar explant.

We are at present attempting to induce embryoids in the nucellus of unpollinated, unfertilised ovules of the Ellendale mandarin, a monoembryonic variety of commercial importance in South Africa.

We are also attempting to find and identify the active fraction of ME, and are carefully testing the efficacy of AD and AS, since AD appeared to be more effective even when the equivalent amount of AD was applied in the sulphate form.

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